Lack of Evidence of ACE2 Expression and Replicative Infection by SARSCoV-2 in Human Endothelial Cells

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Lack of Evidence of ACE2 Expression and Replicative Infection by SARS-CoV-2 in Human Endothelial Cells

Running Title: *McCracken, Saginc, He, Huseynov, et al.; Absence of ACE-2 Limits Endothelial Cell Infection*

Ian R. McCracken, BSc (Hons)[1*]; Gaye Saginc, PhD[2*]; Liqun He, PhD[3,7*]; Alik Huseynov, PhD[2*]; Alison Daniels, BSc (Hons)[4$]; Sarah Fletcher, PhD[5$]; Claire Peghaire, PhD[2]; Viktoria Kalna, PhD[2]; Maarja Andaloussi-Mäe, PhD[3]; Lars Muhl, PhD[6]; Nicky M. Craig, PhD[5]; Samantha J. Griffiths, PhD[4]; Jürgen G. Haas, MD, PhD[4]; Christine Tait-Burkard, PhD[5]; Urban Lendahl, PhD[6,8]; Graeme M. Birdsey, PhD[2^]; Christer Betsholtz, PhD[3,6,9^]; Michela Noseda, MD, PhD[2^]; Andrew H. Baker, PhD[1^]; Anna M. Randi, MD, PhD[2^]

[1]Centre for Cardiovascular Science, University of Edinburgh, Little France Crescent, Edinburgh, UK; [2]National Heart and Lung Institute, NIHR Imperial Biomedical Research Centre, Imperial College London, London, UK; [3]Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden; [4]Infection Medicine, University of Edinburgh, Little France Crescent, Edinburgh, UK; [5]The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK; [6]Integrated Cardio Metabolic Centre, Karolinska Institute, Huddinge, Sweden; [7]Department of Neurosurgery, Tianjin Medical University General Hospital, Tianjin Neurological Institute, Key Laboratory of Post-Neuroinjury Neuro-Repair and Regeneration in Central Nervous System, Ministry of Education and Tianjin City, Tianjin 300052, China; [8]Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden; [9]Department of Medicine Huddinge, Karolinska Institute, Huddinge, Sweden

[*]These authors share first Authorship
[$]These authors contributed equally
[^]These authors share senior authorship

Addresses for Correspondence:

Anna M. Randi, MD, PhD
National Heart and Lung Institute
Vascular Sciences, Imperial College London
Hammersmith Hospital, Du Cane Road
London, W12 0NN, UK
Tel: +4420 7594 2721
Email: a.randi@imperial.ac.uk

Andrew H. Baker, PhD
Centre for Cardiovascular Science
University of Edinburgh
47 Little France Crescent
Edinburgh, EH16 4TJ, UK
Tel: +44131 242 6728
Email: Andy.Baker@ed.ac.uk

A striking feature of severe forms of coronavirus disease 2019 (COVID-19), the current pandemic caused by the coronavirus SARS-CoV-2, is severe endothelial injury with micro- and macro-thrombotic disease in the lung and other organs, including the heart. This has led to speculation that viral infection may damage the endothelium through two mechanisms: indirectly, via neighbourhood effects, circulating mediators and immune mechanisms, or directly by viral infection of endothelial cells (EC).

To support the hypothesis of direct viral damage of EC via virus-induced infection, the cells should express the main receptor for SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2), a metalloproteinase component of the renin-angiotensin hormone system and a critical regulator of cardiovascular homeostasis[1]. Indeed, several recent review articles propose that SARS-CoV-2 binding to ACE2 on EC is the mechanism through which the virus may cause direct endothelial damage and endothelialitis[1]. However, expression of ACE2 in EC has not been convincingly demonstrated to support this assumption, nor has there been sufficient evidence to support a direct infection of EC by SARS-CoV-2.

To address the questions of ACE2 expression in human EC and of the ability of SARS-CoV-2 to infect the endothelium, we interrogated transcriptomic and epigenomic data on human EC and studied the interaction and replication of SARS-Cov-2 and its viral proteins with EC *in vitro*. The data, analytic methods, and study materials will be maintained by the corresponding author and made available to other researchers on reasonable request.

Analysis of RNA-seq was carried out on ENCODE data from EC from arterial, venous and microvascular beds, in comparison with epithelial cells from respiratory, gastrointestinal and skin sources. Very low or no basal *ACE2* expression was found in EC, compared to epithelial cells (Figure A-B). Moreover, *in vitro* exposure of EC to inflammatory cytokines reported as

elevated in the plasma of patients with severe COVID-19 failed to upregulate ACE2 expression (Figure C).

Publicly available single-cell RNA-sequencing (scRNAseq) of human organ donor hearts[2] showed that while *ACE2* sequence reads are abundant in pericytes (PC), they are rare in EC (Figure D). Out of 100,579 EC, only 468 (0,47%) were *ACE2*$^{+}$, and in the majority (424) only a single *ACE2* transcript was detected. This could reflect true low and rare endothelial *ACE2* expression, but also contamination from adherent PC fragments, a common confounder in vascular scRNAseq data[3]. If such fragments contributed the *ACE2* transcripts observed in certain EC, we would expect to detect other pericyte transcripts in the same cells. Indeed, among the top-50 gene transcripts enriched in *ACE2*$^{+}$ vs. *ACE2*$^{-}$ EC, we noticed several known pericyte markers, including *PDGFRB, ABCC9, KCNJ8* and *RGS5* (Figure E). Comparison of transcript abundance across the three major vascular and mesothelial cells showed that the top-50 gene transcripts were expressed at the highest levels in PC (Figure E). This suggests that the rare occurrence of *ACE2* transcripts in human heart EC is likely caused by pericyte contamination. Similar conclusions have previously been reached in mouse tissues[3].

Analysis of the chromatin landscape at the *ACE2* gene locus in human umbilical vein EC (HUVEC) using data from ENCODE further supports this concept. The histone modification mark H3K27me3, which indicates repressed chromatin, was enriched at the *ACE2* transcription start site (TSS); conversely, promoter, enhancer and gene body activation marks (H3K27ac, H3K4me1, H3K4me2, H3K4me3, H3K36me3), RNA polymerase-II and DNase I hypersensitivity were absent or low, suggesting that *ACE2* is inactive in EC. In marked contrast, the adjacent gene *BMX*, an endothelial-restricted non-receptor tyrosine kinase displays an

epigenetic profile consistent with active endothelial expression (Figure F). Thus, transcriptomic and epigenomic data indicate that *ACE2* is not expressed in human EC.

Other cell surface molecules have been suggested as possible receptors for the virus, but their role in supporting SARS-CoV-2 cell infection remains to be demonstrated. We therefore tested directly whether EC could be capable of supporting coronavirus replication *in vitro*. Productive levels of replication in primary human cardiac and pulmonary EC were observed for the human coronavirus 229E GFP reporter virus[4], which utilises CD13 as its receptor, demonstrating directly that human EC can support coronavirus replication in principle (Figure G). However, when cells were exposed to SARS-CoV-2, replication levels were extremely low for EC, even following exposure to very high concentrations of virus compared to more permissive VeroE6 cells (Figure H). The observed low levels of SARS-CoV-2 replication in EC are likely explained by viral entry via a non-ACE2 dependent route, due to exposure to extremely high concentrations of virus in these experiments (MOI 10 and 100).

These data indicate that direct endothelial infection by SARS-Cov-2 is not likely to occur. The endothelial damage reported in severely ill COVID19 patients is more likely secondary to infection of neighbouring cells and/or other mechanisms, including immune cells, platelets and complement activation, and circulating proinflammatory cytokines**.** Our hypothesis is corroborated by recent evidence that plasma from critically ill and convalescent patients with COVID-19 causes endothelial cell cytotoxicity[5]. These finding have implications for therapeutic approaches to tackle vascular damage in severe COVID19 disease.

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Conflict of Interest Disclosures

None

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For ENCODE database, please see www.encodeproject.org.

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Figure Legend

Figure. Analysis of *ACE2* expression in human endothelial cells and of coronavirus replication in primary human endothelial cells.

(A-B) Comparison of *ACE2* expression in human primary epithelial and endothelial cells using total RNA-seq data from the ENCODE Database shows low or absent expression in EC. (A) The difference of *ACE2* expression in epithelial and endothelial cells is shown in boxplots with individual, as well as grouped samples (inner boxplot). Each dot represents a single sample (n=2 per cell type). (B) Transcriptome profiles of epithelial and endothelial cells are shown in a density plot, using the median of all samples per group (n=19360 genes). *ACE2* expression in each group is marked with a dotted line: *ACE2* expression in endothelial cells (red) overlaps with the peak for non-expressed transcripts (highlighted in grey), while *ACE2* expression in epithelial cells (blue) is to the right, indicating detectable expression. Median *ACE2* expression in endothelial cells equals -5.6 in log2 CPM (Counts Per Million), which corresponds to 0 raw read counts, signifying undetectable *ACE2* expression in the majority of endothelial cells. Expression values in all plots are represented as log2-transformed CPM, normalized by Trimmed Mean of M-value (blue: epithelial, red: endothelial). **(C) *ACE2* expression is not regulated by inflammatory cytokines in HUVEC.** qPCR analysis of *ACE2* mRNA expression in HUVEC treated with a mix of 4 cytokines/chemokines (TNF-α, IL1-β, IL8 and IL6/IL6R chimeric protein) for 4h or 24h at 0, 0.01, 0.1 or 1.0 ng/ml. Data are normalized to *GAPDH* and presented as mean ± SEM of 3 independent experiments. **(D-E) Very low-level, rare and likely contaminating *ACE2* transcripts are seen in EC.** (D) *ACE2* transcript reads are detected preferentially in PC. UMAP landscapes of publicly available human heart datasets[2]

include 100,579 endothelial cells (EC), 77,856 pericytes (PC), 16,242 smooth muscle cells (SMC) and 718 mesothelial cells (MC) (https://www.heartcellatlas.org/). *ACE2* transcript reads are detected preferentially in the PC cluster (enriching for *ABCC9*) and are rare in the EC cluster (enriching for *PECAM1*). (E) PC transcripts are enriched together with *ACE2* in 0.47% of EC. Dot plot displaying the abundance of top-50 transcripts enriched *ACE2*+ vs. *ACE2*- EC, across cell types indicated in D. (The Wilcoxon Rank Sum tests with Bonferroni-corrected p values are < 1E-60 for each). **(F) Epigenetic profiling indicates that the *ACE2* gene is inactive in EC.** ChIP-seq binding profiles in HUVEC for histone modifications, RNA Pol2 enrichment and DNAse I hypersensitivity. The x axis represents the genomic position, the transcription start sites are indicated by closed arrows and the direction of transcription is indicated by open arrows; the y axis shows ChIP-seq signal in reads per million per base pair (rpm/bp). The bottom row represents the chromatin state segmentation. Colour key: active promoter, red; enhancers, yellow; transcriptional elongation, green; repressed, grey. **(G-H) Coronavirus replication in primary human cardiac and pulmonary endothelial cells shows limited replication of SARS-CoV-2.** (G) Viral replication curves in human pulmonary (HPAEC) and cardiac (HCAEC) endothelial cells following infection with control HCoV-229E GFP reporter virus (MOI = 0.6). Virus replication was measured via GFP fluorescence every 2 hours from 20 to 58 hours post inoculation. Mean ±SEM of 3 technical replicates are shown at each time point for each biological replicate. (H) Viral growth curves in HPAEC (n=3), HCAEC (n=3), and non-endothelial Vero cells (n=1) following infection with SARS-CoV-2 at MOI = 10 or 100. Supernatant were collected at 1, 24, and 48 hours post infection and virus copy number quantified by RT-qPCR detection of the SARS-CoV-2 N3 gene. TCID= tissue culture infectivity dose.